EXHIBIT F

IMMUNOLOGY

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The de novo pathway, in which a methyl or formyl group is transferred from an activated form of tetrahydrofolate, is blocked by *aminopterin*, a folic acid analog. When the de novo pathway is blocked, cells utilize the salvage pathway, which bypasses the aminopterin block by converting purines and pyrimidines directly into DNA. The enzymes catalyzing the salvage pathway include hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK). A mutation in either of these two enzymes blocks the salvage pathway. HAT medium contains *a*minopterin to block the de novo pathway and *h*ypoxanthine and *t*hymidine to allow growth via the salvage pathway. When two types of cells, each of which has a mutation in a different enzyme necessary for the salvage pathway, are fused, only the hybrid cells will contain the full complement of the necessary enzymes for growth on HAT medium via the salvage pathway. Culture in HAT medium thus allows only the hybrid cells to grow.

Production of Monoclonal Antibodies

The production of a given monoclonal antibody involves three basic steps: (1) generating B-cell hybridomas by fusing primed B cells and myeloma cells; (2) screening the resulting clones for those which secrete antibody with the desired specificity; and (3) propagating the desired hybridomas.

Generating B-Cell Hybridomas

In their innovative method for producing monoclonal antibodies, Kohler and Milstein applied the techniques of cell fusion and HAT selection of hybrid cells described in the previous section. Their general procedure is outlined in Figure 7-3. The use of myeloma cells that cannot grow in HAT medium (HGPRT$^-$ cells) assured that only hybridomas (hybrid myeloma-spleen cells) were selected. The unfused or fused spleen cells did not need

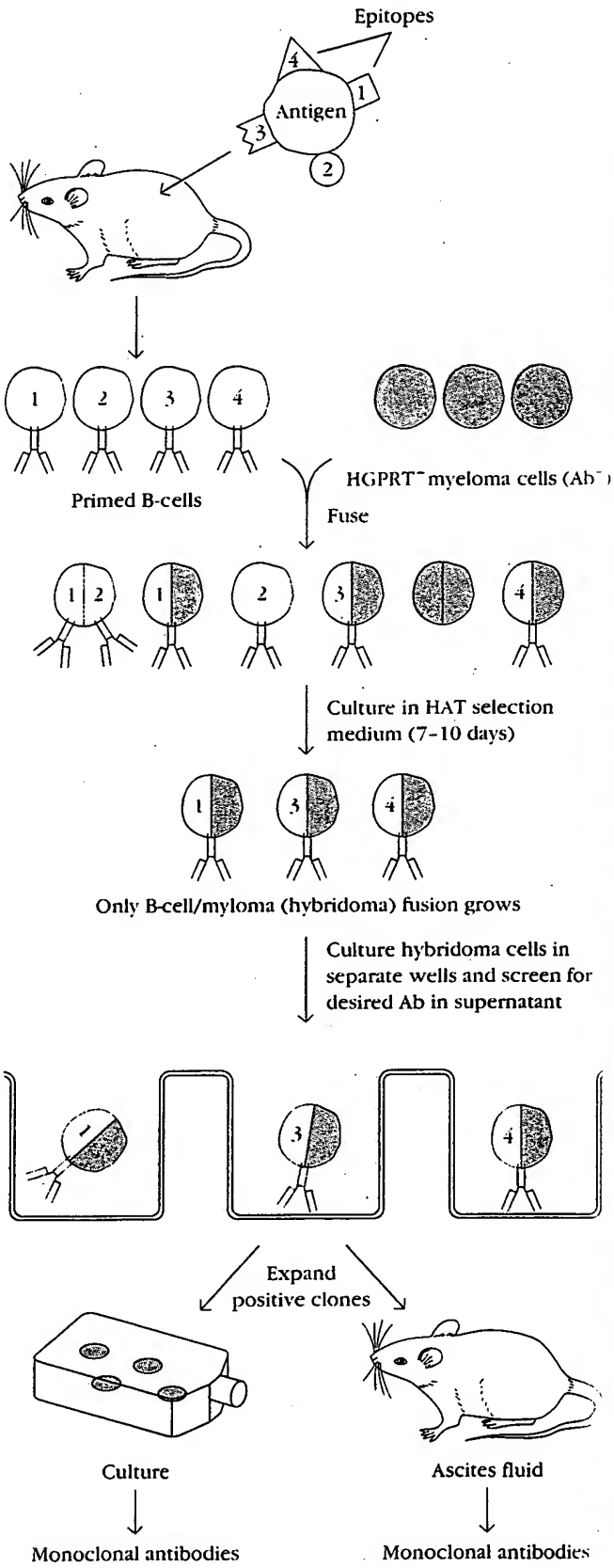


Figure 7-3 The procedure for producing monoclonal antibodies specific for a given antigen developed by G. Kohler and C. Milstein. Spleen cells from an antigen-primed mouse are fused with mouse myeloma cells (HGPRT$^-$ and Ab$^-$). The spleen cell provides the necessary enzymes for growth on HAT medium, while the myeloma cell provides immortal-growth properties. Unfused myeloma cells or myeloma-myeloma fusions fail to grow due to lack of HGPRT. Unfused spleen cells have limited growth and therefore do not need an enzyme deficiency for elimination with the HAT selection procedure.

to be selected because they were terminal cells of a differentiation series and were only capable of limited growth in vitro. After 7–10 days of culture in the HAT medium, most of the wells contained dead cells, but a few wells contained small clusters of viable cells, which could be visualized by using an inverted phase contrast microscope. Each cluster represented clonal expansion of a hybridoma (Figure 7-4). After HAT selection, single cells were transferred and cultured in separate wells in an effort to ensure the monoclonality of any secreted antibody. Wells containing single viable clusters were then screened for antibody production; antibody-positive clones were subcultured at low cell densities, again to ensure clonal purity in each microwell. The hybridoma clones obtained by this procedure were isolated, clonally expanded in culture, and shown by Kohler and Milstein to produce monoclonal antibodies, each specific for a single epitope on sheep red blood cells, the original antigen used in their experiments.

The first hybridomas obtained by Kohler and Milstein secreted not only antibody from the splenic B cell but also unwanted antibody from the myeloma cell as well as some hybrid antibody combining heavy or light chains from both original parent cells. To avoid this difficulty, an HGPRT$^-$, Ab$^-$ myeloma cell was chosen as the ideal fusion partner. This fusion partner has the immortal-growth properties of a cancer cell but does not secrete its own antibody gene product. Hybridomas generated with this fusion partner thus secrete only the antibody from the B-cell partner. These hybridomas can be propagated in tissue culture to give rise to large clones secreting homogeneous monoclonal antibody.

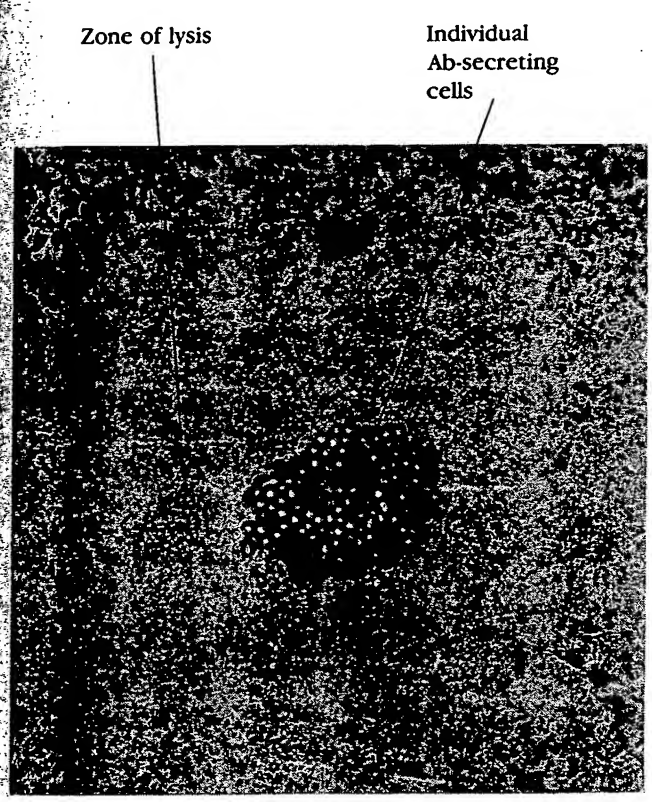
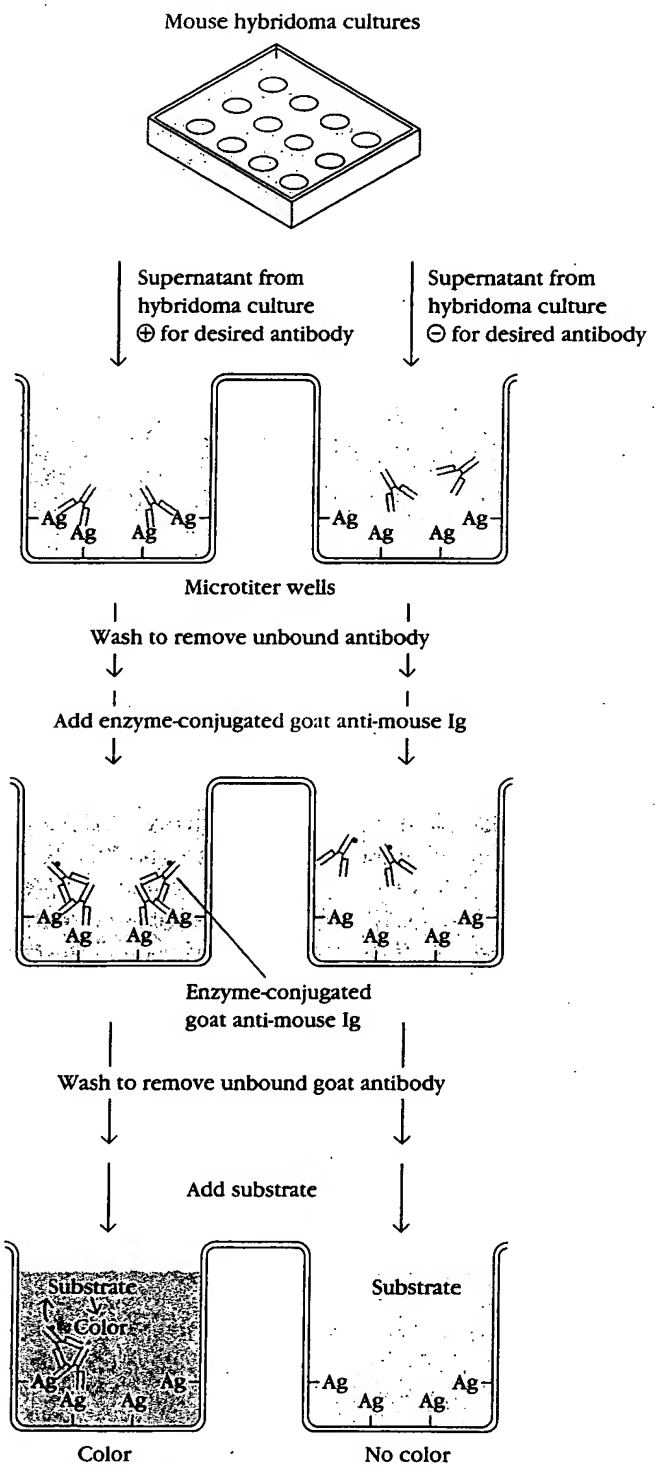


Figure 7-4 Viable hybridoma clone that appeared after culture for 7–10 days in HAT selection medium. The individual cells in the clone are visible. This particular clone secreted monoclonal antibody to sheep red blood cells (SRBCs). When SRBCs and complement were added, the anti-SRBC antibody diffusing out from the secreting cells caused complement-mediated lysis of the red blood cells, producing the visible clear zone. [From C. Milstein, 1980, *Sci. Am.* 243:67.]

Screening for Monoclonal Antibody Specificity

Once pure clones of antibody-secreting hybridomas are obtained, they must be screened for the desired antibody specificity. Although some hybridomas will produce antibody specific for the antigen used for immunization, others will be specific for unwanted antigens. The supernatant of each hybridoma culture contains its secreted antibody and can be assayed for a particular antigen specificity in various ways. Two of the most common screening techniques are ELISA and RIA, both of which are easily adapted to mass screening with 96-well microtiter plates. In both assays, antigen that reacts with the desired antibody is bound to the microtiter wells and washed to remove unbound antigen. Supernatant from each hybridoma well is added to separate wells. After incubation and more washing, an anti-isotype antibody directed against the isotypic determinants on the monoclonal antibody is added. In an ELISA this anti-isotype antibody is conjugated to an enzyme that produces a colored reaction product when the appropriate substrate is added (Figure 7-5). In an RIA the anti-isotype antibody is radiolabeled; bound label can be detected by counting the wells individually in a gamma counter, or the entire plate can be exposed to x-ray film. If the desired monoclonal antibody has specificity for a cell-membrane molecule, immunofluorescent techniques can be used for screening. In this case, target cells with the particular cell-membrane antigen are stained with the monoclonal antibody in microtiter wells and visualized by the addition of a fluorochrome-conjugated anti-isotype antibody (see Figure 6-15). Alternatively, a fluorescence-activated cell sorter can be modified to microsample labeled target cells taken from the microtiter wells.



Propagating Hybridomas Secreting Specific Monoclonal Antibodies

Once a hybridoma secreting a monoclonal antibody of the desired specificity has been identified, it should be recloned by limiting dilution to ensure that the culture is truly monoclonal. The cloned hybridoma can then be propagated in one of several ways to produce the desired monoclonal antibody. The hybridoma can be grown in tissue-culture flasks, in which the antibody is secreted into the medium at fairly low concentrations (10–100 μg/ml). The hybridoma also can be propagated in the peritoneal cavity of histocompatible mice, where it secretes the monoclonal antibody into the ascites fluid at much higher concentrations (1–25 mg/ml); the antibody can then be purified from the mouse ascites fluid by chromatography.

To meet the increased demand for monoclonal antibodies, biotechnology companies have been developing various techniques to increase yields. Damon Biotech Company encapsulates hybridomas in alginate gels, which allow nutrients to flow in and waste products and antibodies to flow out. In these capsules, hybridoma cells can achieve much higher densities than in tissue culture; as a result, 100-fold greater yields of antibody production have been attained by this method than with conventional tissue culture. A different approach has been taken by Celltech in England. In this company's method, hybridomas are grown in 1000-liter fermenters, which yield 100 grams of monoclonal antibody in a 2-week period. Further scale-ups to 10,000-liter fermenters are being developed.

Figure 7-5 ELISA screening of mouse hybridomas for those secreting monoclonal antibody of desired specificity. Microtiter wells are coated with the desired antigen, and supernatant from each hybridoma culture is added to a well. After incubation to allow antibody to bind, the unbound antibody is washed away. An enzyme-conjugated goat anti-mouse antibody is then added. This anti-isotype antibody will bind to the mouse monoclonal antibody. The unbound goat antibody is washed away and a substrate for the conjugated enzyme is added. If the original supernatant contains antibody specific for the antigen, a colored reaction product will be formed on addition of substrate (*left*). The absence of color (*right*) indicates that the tested hybridoma does not secrete the desired antibody.